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GRANT NUMBER DAMD17-94-J-4230

TITLE: Homeobox Genes in Normal, Preneoplastic, and Neoplastic Mammary Glands

PRINCIPAL INVESTIGATOR: Charles W. Daniel, Ph.D.

CONTRACTING ORGANIZATION: University of California
Santa Cruz, California 95064

REPORT DATE: January 1997

DTIC QUALITY INSPECTED 2

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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19970610 017

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 1997	Annual (1 Jan 96 - 31 Dec 96)

4. TITLE AND SUBTITLE
Homeobox Genes in Normal, Premeoplastic and Neoplastic Mammary Gland

5. FUNDING NUMBERS
DAMD17-94-J-4230

6. AUTHOR(S)
Charles W. Daniel, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Santa Cruz, California 95064

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The second year of funded research has been productive and exciting. In the mouse, we have studied the expression of Hoxa-2. Like Hoxa-1, it is not expressed in the normal mammary gland or in precancerous lesions, but is heavily expressed in many cancers. This suggests a role is the later and more malignant stages of tumor progression. As part of a study to explore expression of homeotic genes in the human breast, we have discovered, cloned, and partially characterized a large new family of homeobox-containing genes, which we have termed IRX (Iroquois Related). This family, which consists of at least six new genes, contains a DNA binding homeobox that is only distantly related to the HOX genes, but which has close homology with a recently discovered family in Drosophila, the IROC (Iroquois) complex. In the human, IRX-2 is abundantly expressed in the lumenal mammary epithelium, but not in myoepithelial or stromal tissues. In breast cancers the gene is often misexpressed. The discovery of the IRX family opens the door to a new and unexpected area of investigation, with exciting possibilities for both normal and malignant aspects of breast development.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Hox, Homeobox, Hormone, Precancerous, Growth Factors, Malignant Progression, Humans, Anatomical Samples, Mammary, Breast Cancer	33
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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[signature] Jan 27, 1997

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INTRODUCTION

In 1996 the highlight of our research is undoubtedly the discovery of a new, large family of homeotic genes in the human breast. These genes were cloned, partially characterized, and their expression has been studied. They are activated in lumenal breast epithelial cells, but not in myoepithelium or in stroma. This highly specific localization is also found in breast cancers, although the pattern of expression is much more variable. We feel that the discovery of the IRX family of homeogenes will open the door to a wealth of interesting studies in the mammary gland and in embryogenesis.

The discovery of the IRX family is directly in line with the general problem addressed by this grant, now entering its third year of funding, which is to obtain a better and ultimately more clinically useful understanding of the genetic mechanisms underlying development of the normal breast and of the initiation, progression, and spread of breast cancer. The reasoning behind this question is as follows. The breast is a target organ for a variety of hormones. These, together with growth/differentiation factors, regulate the activities of the mammary cell. Unfortunately, this does not take us very far in understanding the biology of this interesting organ. Consider simply that other organs are also regulated by these same signaling molecules, but develop by quite a different pattern. The mammary gland itself varies enormously between species, between individuals, and of course in malignancy. How can this variation be accounted for, when the signals are the same? There must exist additional layers of genetic regulation that interpret these signals and give rise to particular patterns of development, or to neoplasias. How do we search for these developmental regulatory genes? In organisms such as *Drosophila*, where detailed genetic analysis is possible, mutations provide clues that have led geneticists to identify gene families that act as master regulators of cell fate, determining for example, whether a wing or an antenna will develop at a particular location. The discovery of these regulators has had an enormous impact on thinking in biology.

One such group of genes is the homeotic family, which act as transcription factors, switching on or off groups of genes that specify the details of developmental processes. One of the triumphs of molecular biology has been the discovery that these "homeobox" genes are not limited to the fly, but are ubiquitously distributed and remarkably conserved. In the mammals, including the human, not only do they occur in the genome, but their numbers and types are greatly amplified. It is now well established in the mouse that these genes are essential for determining many aspects of early embryogenesis, and more recently, it seems that they may be active in the development of tissues and organs, and that their malfunctions may contribute to cancer.

Do homeobox genes influence the breast, and if so, could they contribute to cancer? This is the question underlying our research, and during the first year we have discovered that many of these homeobox genes are active during the growth and development of both the mouse mammary gland and human breast. Their patterns of expression are frequently altered in cancer, some overexpressed, others underexpressed or not expressed at all. Because these genes serves such central regulatory roles, these discoveries create optimism that new insights into mammary development will be a product of these studies, and that an entirely new class of mammary oncogenes or tumor suppresser genes may exist.

Our approach is to use the mouse mammary tumor model system for those observations and experiments that describe the patterns of expression of these genes. In addition, the mouse model, with its capability of "reverse genetics," provides the potential to explore the role of homeotic genes in mammary development and in the etiology of cancer. These experiments cannot be done in humans, of course, but we are simultaneously

carrying out expression studies on normal and malignant breast samples and comparing them with findings obtained with the mouse. In addition, we are cloning human homeobox genes that cannot be obtained elsewhere, and it was in connections with this that the previously mentioned IRX family was discovered.

BODY OF NARRATIVE

During the second year of DAMD support we have brought in new personnel, obtained valuable samples of human breast tissues through collaborative agreements with local hospitals, and made steady progress in the specific aims outlined in the original proposal. One paper has been published during this year (Friedmann and Daniel, 1996) and another submitted. We are particularly pleased and excited about Aim No. 2, in which we are studying the expression of homeobox genes that may influence breast development and breast cancer. As seen below, we have discovered, cloned, and characterized members of a large new family of homeotic genes in the human. This gene family will doubtless have important implication to human embryological development, but of immediate interest to us is the robust, highly localized expression in mammary epithelial cells and their malignant counterparts.

We continue to be pleased that this research has attracted widespread attention, as judged by requests for reprints, applications for postdoctoral positions, and invitations to present papers at national and international meetings. I continue to hope that several other laboratories will become engaged in homeogene research in the breast, and progress will accelerate.

All of our data continue to indicate that homeobox genes are expressed in the development of mammary glands in both mice and humans. More important, the spatial and temporal pattern of this expression indicates a regulatory role for this interesting family of transcription factors, in which altered expression patterns may contribute to various morphotypes, and to cancer initiation or progression. We continue to be optimistic that we have uncovered a new level of genetic regulation of breast growth, morphogenesis, and function, and that at least some of this large family of genes may be shown to constitute a new class of mammary oncogenes.

Our progress in the four areas identified as research objectives is summarized below:

Objective 1. *Do expression patterns suggest a role for homeobox genes in mammary growth and development in the mouse? What is the pattern of homeobox gene expression in precancerous tissue and in malignancies?*

Inventory of clones, probes, and antibodies. Essential tools for investigating homeogene expression in the mammary gland are molecular clones, enabling temporal analysis by northern hybridization and spatial distribution by *in situ* hybridization. Antibodies are especially powerful because they provide information on spatial localization of homeoproteins, thus obviating the possibility that a gene may be transcribed but not translated into a functional product. Because the numbers of homoebox genes is very large, building an useful inventory of these reagents is a daunting task, and is accomplished only by persistent effort over a period of time. Table 1 gives our present inventory, and documents our ability to make progress along a much broader front than was previously the case.

TABLE 1: HOMEOBOX CLONE SUMMARY

HUMAN							MOUSE	
CLONE ID	GENBANK IDENT.	CLONE SOURCE	CLONE OBTAINED?	CLONE IDENTITY CORRECT?	PRESUMTIVE IDENTITY (IMAGE/TIGR)	ANTIBODY AVAIL.	CLONE OBTAINED?	CLONE IDENTITY CORRECT?
					HOX A1	YES	YES	YES
					HOX A2	YES	YES	YES
210731	H66899	IMAGE	YES	YES	HOX A4	YES		
105250	T29089	ATCC			HOX A5	YES		
p15.10 et. al		PCR (R1+L1)	YES	YES	HOX A7	YES		
126701	R07021	IMAGE	YES	NO	HOX A10	YES		
					HOX B1	YES		
					HOX B2	YES		
					HOX B3	YES		
					HOX B4	YES		
150702	H02340	IMAGE	YES	YES	HOX B5			
					HOX B6	YES		
HOX 2C		ANABELLA	YES	YES	HOX B7	YES	YES	YES
					HOX B8	YES	YES	YES
					HOX B9	YES	YES	YES
					HOX C4	YES		
101779	T28272	ATCC	TO BE ORDERED		HOX C5			
HOX 3C		ANABELLA	YES	YES	HOX C6	YES	YES	YES
					HOX C8	YES	YES	YES
1:4A		PCR (R1+AP1)	YES	YES	HOX C10			
					HOX C13		YES	YES
251373	H96751	IMAGE	YES		HOX D1			
					HOX D3		YES	YES
HOX 4B		ANABELLA	YES	YES	HOX D4	YES	YES	YES
51592	H24026	IMAGE	YES	NO	HOX D4			
107563	T29810	ATCC	TO BE ORDERED		HOX D8		YES	YES
249133	H82729 H83603	IMAGE	YES		HOX D9		YES	YES
HOX 4.5		DUBOULE	YES	YES	HOX D10	YES	YES	YES
					HOX D11		YES	YES
					HOX D12	YES	YES	YES
HOX 7		PADANILAM / MURRAY	YES	YES	MSX-1		YES	YES
192157	H40539	IMAGE	YES	YES	MSX-2		YES	YES
					EVX-1		YES	YES
					EN-1		YES	YES
					EN-2		YES	YES
C-34C02	F11981	IMAGE	YES	YES	DLL			
50354	H17803	IMAGE	YES	YES	OTX-2			
161675	H25643	IMAGE	YES	YES	MEIS-2/BHD-1			
IRX-1		RACE PCR	YES	YES	IRX-1/L7.7			
152453	R46202	IMAGE/RACE PCR	YES	YES	IRX-2/BHD-2			
IRX-3		RACE PCR	YES	YES	IRX-3/L7.2,4			
IRX-4		RACE PCR	YES	YES	IRX-4/L7.3			
IRX-5		RACE PCR	YES	YES	IRX-5/L7.5			

Msx-1 and *Msx-2*

In the Progress Report for 1995 our experiments with two divergent homeobox genes, *Msx-1* and *Msx-2* were reported. These studies have been confirmed, extended, and are recently published (Friedmann and Daniel, 1996). Because our most recent experiments did not alter the conclusions previously reported, rather than repeat these data I shall summarize the salient points concerning this interesting gene family and their possible role in the mammary gland, and refer the reader to the reprint (Appendix).

Msx-1 and *Msx-2* are related to the *Drosophila melanogaster* muscle segment homeobox (*msh*) gene, that contains a homeobox which is markedly divergent from that of any other characterized *Drosophila* genes. In *Drosophila*, *msh* is mainly expressed in the central nervous system and in segmented striated muscles of the body wall. In the mouse there appear to be three distinct msh-like genes, named *Msx-1, Msx-2* and *Msx-3*, which are found at separate loci and are not clustered (Hill et al., 1989; Robert et al., 1989; Monaghan et al., 1991; Holland, 1991). Closely related versions of *Msx-1* and *Msx-2* have been identified in a variety of vertebrate species including Zebrafish (Ekker et al., 1992), Xenopus [Su, 1991], and the chick (Coelho et al., 1991). later patterns of gene activation have been examined by *in situ* hybridization methods in the development of several organs, including the mouse and chick limb bud (Nohno et al., 1992; Davidson et al., 1991; Robert et al., 1991), mouse tooth bud (Mackenzie et al., 1991a; MacKenzie et al., 1992; Jowett et al., 1993), chick heart (Chan-Thomas et al., 1993) and chick craniofacial development (Nishikawa et al., 1994). The results suggested that the two genes play a role in epithelial-mesenchymal interactions in these developing organs.

The expression level of *Msx-1* and *Msx-2* transcripts was evaluated by northern blot hybridization to poly $(A)^+$ enriched RNA isolated from mouse mammary glands at several stages of development. To provide data on spatial localization of transcripts, we have also used *in situ* molecular hybridization using gene-specific probes on sections of mammary gland tissue, as proposed.

1. Like many of the Hox genes, Msx-1 and Msx-2 transcripts were present in glands of virgin mice in early pregnancy, but transcrips decreased dramatically during late pregnancy.
2. Low levels of Msx-1 transcripts were detected in glands from lactating animals and during the first days of involution, whereas Msx-2 expression was not detected during lactation or early involution.
3. Expression of both genes increased gradually as involution progressed.
4. To evaluate the influence of mammogenic hormones on gene expression, we found that Msx-2 but not Msx-1 expression was decreased following ovariectomy, or following exposure to anti-estrogens implanted directly into the gland.
5. Hormonal regulation was confirmed when transcripts returned to normal levels when estrogen was administered to ovariectomized animals.
6. *In situ* molecular hybridization for Msx-1 showed transcripts localized to the mammary epithelium, whereas Msx-2 expression was confined to the periductal stroma.
7. Mammary stroma from which mammary epithelium had been removed did not transcribe detectable amounts of Msx-2, showing that expression is regulated by contiguous mammary epithelium.

These results indicate that expression of these homeotic genes is developmentally regulated, hormonally controlled, and dependent upon epithelial-stromal tissue interaction. The implication for functional roles in mammary development is inescapable, though conclusive evidence must await studies in which gene expression is artificially altered by either transgenic or targeted gene inactivation techniques.

Hoxa-2.

One of the most interesting findings concerning possible roles of homeobox genes in cancer was the discovery that in the mouse, Hoxa-1 is not expressed in either normal mammary gland or precancerous nodules (HAN), but often shows strong expression in tumors derived from these nodules (Friedmann et al, 1994). Thus, Hoxa-1 differed from genes in the other Hox clusters, in which expression was generally found in normal gland, but not in tumors. We have now examined the expression of the contiguous gene in this linkage group, Hoxa-2. Expression is not detectable in normal gland, but substantial levels of transcripts were seen in two of three tumors examined (Fig. 1).

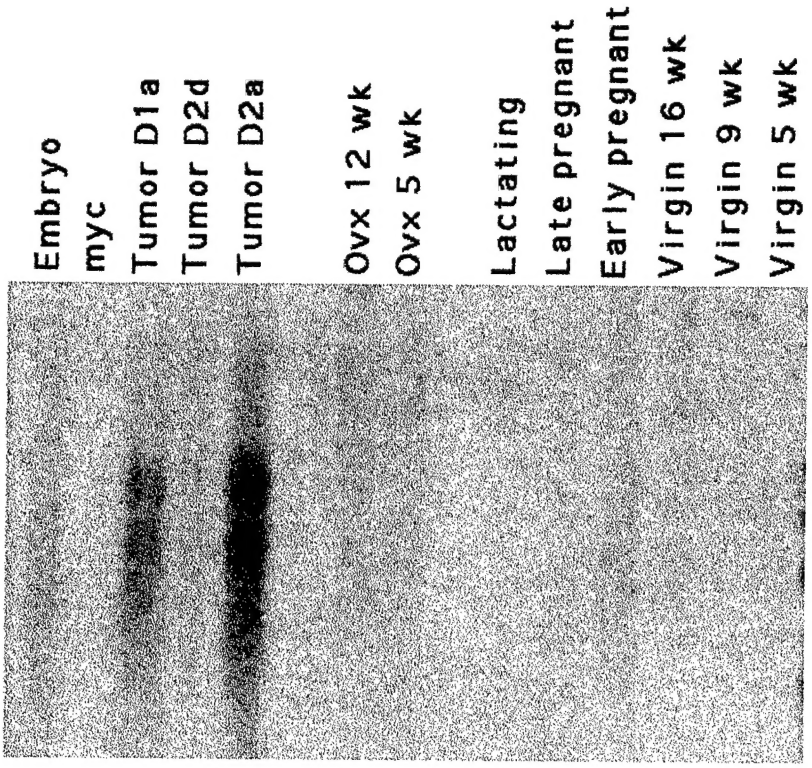


Figure 1. Northern hybridization of a gene-spoecific probe to Hoxa-2 showing expression in various developmental stages of the mammary cycle, and in tumors.

This pattern of expression appears to be characteristic of the 5' members of the Hoxa linkage group, and of course we intend to continue to examine other family members. With respect to both Hoxa-1 and Hoxa-2, it can be said the their role in cancer is most likely to be in later stages of tumor progression, rather than earlier, perhaps involving changes associated with malignancy such as invasivness, metastasis, or angiogenesis. In the case of Hoxb, Hoxc, and Hoxd genes, we have postulated that their role in the normal

gland is to drive cells towards the differentiated phenotype, and under expression in cancer may have the effect of maintaining the mammary cells in a proliferating state, permitting other genetic alterations to accrue. In the case of Hoxa the opposite may be the case, and expression may be associated with proliferation of relatively undifferentiated cells.

Objective 2. *Using human tissues obtained from mastectomy and reduction mammoplasty patients, we can ask if homeobox genes are expressed in human breast tissues. Are breast cancers associated with altered levels of expression?*

In research with primary human tissues, success or failure may revolve on the cooperation between the surgeons and research personnel. This is particularly true when, as in the present case, success depends upon the tissue being specially treated by immediate freezing in liquid N_2 or by immersion in ice-cold paraformaldehyde fixative. We are fortunate in having the cooperation of surgeons who are committed to this research and who follow our protocols with care. We are collecting tissue on a regular basis, and adequate numbers of samples will not present a problem.

As described in the previous progress report, our initial strategy to investigate homeobox genes in the human breast was to prepare cDNA from tissue samples and amplify fragments using degenerate primers to conserved homeobox sequences. These fragments have been cloned and sequenced. We identified HOXA4, A7, B4, B7, C6 and D10 by direct sequencing or by hybridization to existing sequence-specific probes. This showed that at least one member of each of the four major HOX clusters is expressed, and we anticipate that there will be many more. In addition, the expression of one homeobox gene outside of the clusters, MSX1 was identified. Again, there will almost certainly be others.

The IRX Complex: a large new family of human homeotic genes expressed in the breast and in breast cancer

To expand our studies into the area of human breast development and breast cancer, we undertook a search for homeobox-containing genes expressed in the breast. We identified five members of a new family of human Iroquois-class homeobox-containing genes (IRX genes) cloned from a human breast cDNA library. We designated these genes IRX after the most closely related class of homeobox genes cloned thus far from Drosophila, the Iroquois complex (IROC) genes araucan (ara) and caupolican (caup) (Gomez-Skarmeta et al., 1996). We have cloned a full-length 1.8 kb cDNA derived from one of these genes, IRX-2, and have characterized its expression by *in situ* hybridization and Northern analysis in several normal and tumor samples. In Northern blot analysis, IRX-2 appears to give rise to at least two transcripts, one 2.6 kb transcript that contains the homeobox and one 3.9 kb transcript that does not. By *in situ* hybridization using a gene-specific probe that recognizes both transcripts, we show that IRX-2 is expressed almost exclusively in lobule-alveolar and lumenal ductal epithelium of the normal breast and not in myoepithelium or periductal stroma. We also show that expression of IRX-2 is maintained in three different tumor types: infiltrating ductal carcinomas (IDC), infiltrating lobular carcinomas (ILC) and fibroadenomas (FA). *In situ* hybridization using a probe that recognizes all closely related homeodomain-containing transcripts suggests that all of these genes are likely to be expressed predominantly in breast epithelial cells. Together, these data indicate that IRX-2 and the related Iroquois-class genes may play a role in establishing and/or maintaining epithelial cell identity in normal and neoplastic breast. Importantly,

continued robust expression and/or misexpression of IRX-2 in mammary tumors and hyperplasias also suggests a possible role in cell proliferation and cancer progression.

Cloning

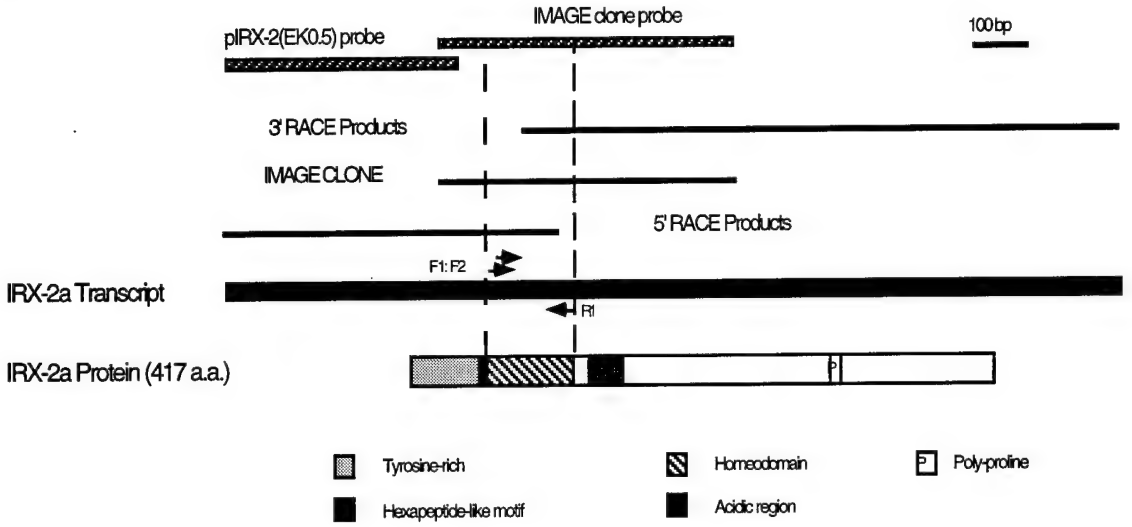
A computer search of nucleotide sequence databases including collections of Expressed Sequence Tags (ESTs) identified a novel homeobox-containing cDNA fragment (as an EST) randomly isolated from a breast cDNA library as a result of the human genome project. This clone (number 152453) was obtained though the IMAGE consortium clone repository and found to be lacking both 5' and 3' ends upon sequencing. Oligonucleotide primers designed to each end of the homeobox (primers F1, F2 and R1) were subsequently used in RACE (Rapid Amplification of cDNA Ends) PCR to isolate the 5' and 3' ends of this cDNA. RACE PCR produced a single major 5' RACE product of about 0.8 kb and several major 3' RACE products of 1.3kb, 0.6kb, 0.5 kb, and 0.3 kb. Preliminary Northern hybridization analysis using the homeobox-containing IMAGE clone probe identified what appeared to be a single transcript at approximately 2.6 kb, therefore the 0.8 kb 5' RACE product and 1.3 kb 3' RACE product were cloned into plasmid vectors and sequenced. Together these two PCR products comprised a full-length 1.8 kb cDNA.

Nucleotide sequence and conceptual translation of IRX-2.

Figure 2 shows the complete nucleotide sequence and conceptual translation of the full length 1.8 kb IRX-2a cDNA. An unusual feature of this cDNA is a relatively high G+C content of 64.4% overall. The high G+C level is maintained over the entire coding region, ranging from about 40% to 90% with only a few short regions dropping below the 60% level (window size=50bp). The 3' untranslated region (3'-UTR) is relatively G+C poor, ranging from about 30% to 50%. The sequences surrounding the presumed initiator methionine ATG codon beginning at position 376 show reasonably good match to the Kozak consensus for translation initiation, though sequences surrounding the second methionine codon (beginning at position 454) are a slightly better match (Kozak, 1987).

The predicted IRX-2a amino acid sequence is 417 amino acids in length and shows a number of interesting features: 1) The homeodomain (residues 49-109) is not closely related to the better known antennapedia-class homeodomain found in the human HOX cluster genes, showing only 33.3 % identity with Antennapedia within the homeodomain. Rather, it is more similar to the Iroquois-class homeodomains encoded by the Iroquois complex (IROC) genes araucan and caupolican (Gomez-Skarmeta et al., 1996), showing 93.4 % identity and 91.8 % identity, respectively (Figure 2). 2) Immediately N-terminal to the homeodomain lies a tyrosine-rich region. The dipeptide motif of a proline followed by a tyrosine (PY) predominates, though other amino acids may substitute for proline. A similar region is also found immediately upstream of the homeodomain in both araucan and caupolican. Within this region, a sequence similar to the core of the hexapeptide motif (consensus 'IYPWMK') found in some homeodomain-containing proteins lies just upstream of the homeodomain and consists of the amino acids 'SYPY'.3) Immediately downstream of the homeodomain is an acidic region, characteristic of some transcription factors and which can act as transcriptional activation domains. 4) The C-terminal portion of IRX-2a downstream of the acidic region is both glycine and proline-rich with a poly-proline motif at positions 254-260. Since both glycine and proline are considered helix-

A



B

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GGAAAAGGGT 360
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M A V E T T V H T H L S A S P P Q G S P
TAC GAC CAC ACA CCC GGC *ATG* GCG GGC TCC TTG GGG TAC CAT CCT TAC GCG GCG CCC CTG GGA TCG TAC CCT TAC 510
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580
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AAG AAC CCC TAC CCC ACC AAG GGC GAG AAG ATC ATG CTG GCC ATC ATC ACC AAG ATG ACC CTC ACC CAG GTG TCC
660
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730
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810
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880
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1260
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1330

C P G P I A G Q A L G G S R A S P A P A P S R S P
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TAC ACG AAC TAT GGC TCC TTC GGA CAC CTT CAT GGC CAC CCG GGG CCC GGG CCA GGC CCC ACA ACC GGT CCG GGG
1480
Y T N Y G S F G H L H G H P G P G P G P T T G P G
TCT CAT TTC AAT GGA TTA AAC CAG ACC GTG TTG AAC CGA GCG GAC GCT TTG GCT AAA GAC CCG AAA ATG TTG CGG 1560
S H F N G L N Q T V L N R A D A L A K D P K M L R
AGC CAG TCT CAG CTA GAC CTG TGC AAA GAC TCT CCC TAT GAA TTG AAG AAA GGT ATG TCC GAC ATT TAA C 1630
S Q S Q L D L C K D S P Y E L K K G M S D I *
GCGGGCTGCG TCGGTCCCGG ACTTTTCTAA TTTATTAAAA ACATGGCCTT GGCAGTTATT TTTCCATCAC CGAGAGAGAG
AGACAGAGAG 1720
AGAAAATAAA CTACCCCTCC TATTCAGAAG TTTATAGTTT ATGGAGATGG ATGACATAAA AATGTAAACA TCTCCACACA
CACAAAAAAA 1810
TGTTTTAACC AACCG(An) 1825

Figure 2: A) Schematic diagram of the IRX-2a cDNA and predicted protein. The extent of the 5' and 3' RACE products relative to the IMAGE clone is shown above the transcript. Postions of the F1, F2, and R1 primers are noted by arrows. Probes used in in situ hybridization and Northern blot analysis are noted above the RACE products. A schematic diagram of the predicted IRX-2 protein is shown below the transcript and relative positions of amino acid motifs are noted. **B)** Nucleotide sequence and conceptual translation of the 1.8 kb IRX-2a cDNA. Presumed initiator methionine codon ATG and second downstream ATG codon are in italics. Sequences corresponding to the Kozak consensus sequence for translation initiation are underlined. Homeodomain amino acids are double underlined.

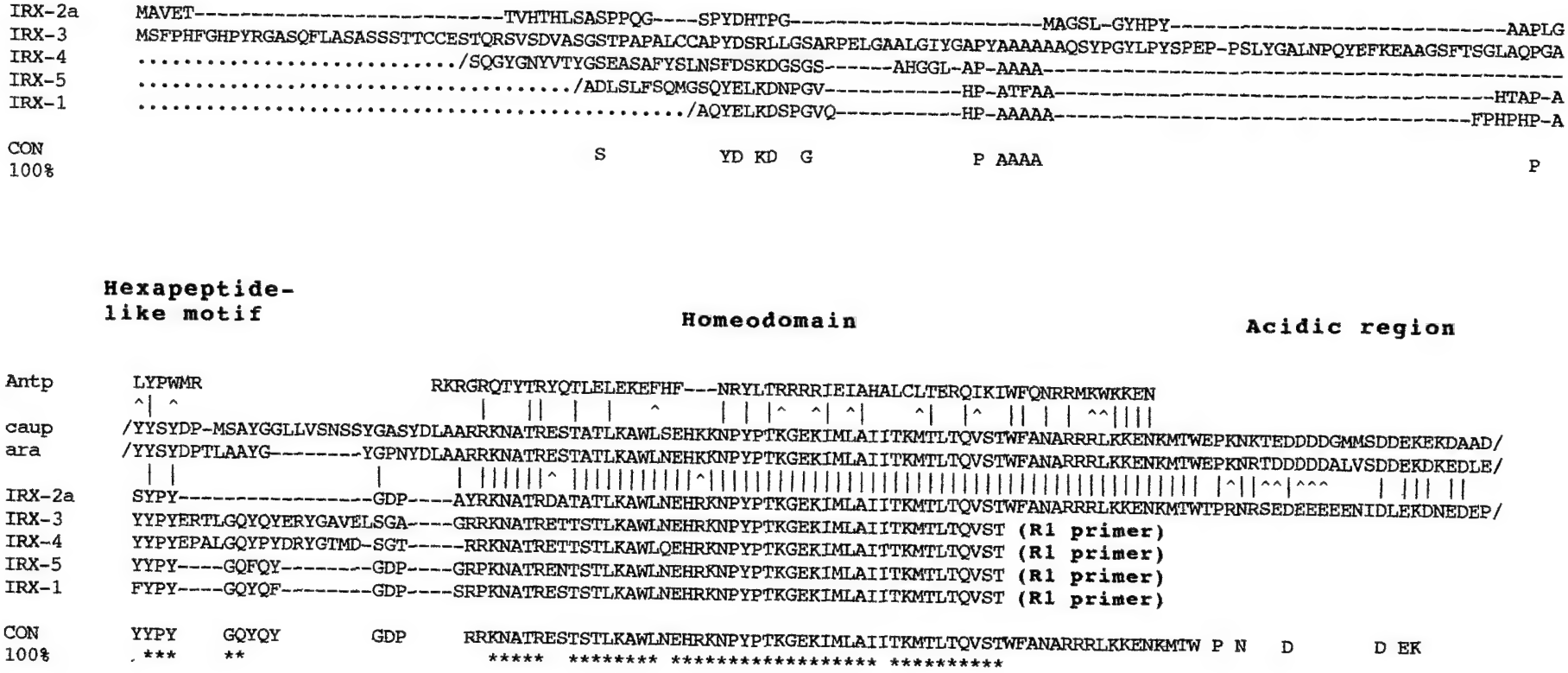

Figure 3. Multiple sequence alignment of predicted IRX proteins translated from 5' RACE products. Predicted IRX protein fragments are shown aligned with equivalent portions of the araucan and caupolican amino acid sequences. Single letter amino acid abbreviations are used. Slashes (/) indicate that the sequences are fragmentary at the end on which they are shown. Gaps used to maximize sequence similarity are denoted by a dash (-). In the comparisons between antennapedia (Antp) and caupolican and between araucan and IRX-2a, vertical lines (|) are used to show amino acid identity and up-angle symbol (^) is used to show amino acid similarity. A consensus sequence is shown below the alignment with amino acids that are 100% conserved designated with an asterisk (*).

breaking residues, secondary structure in this portion of the protein is predicted to be limited to only a few short regions. 5) Unlike both araucan and caupolican, IRX-2 lacks an upstream cysteine-rich EGF-like region.

IRX-2 encodes a member of a large family of Iroquois-class homeodomain proteins

During the course of screening RACE products for IRX-2 cDNAs, we isolated fragments of several related homeodomain-containing transcripts (Figure 3). Each of the transcripts encodes a homeodomain that is, in some cases, virtually identical with that of IRX-2 but diverged dramatically outside of the homeodomain. The designation IRX-1 was given to the cDNA that encoded a homeodomain most similar to that found in the Drosophila Iroquois complex gene ara; the designation IRX-2 was given to the cDNA corresponding to the IMAGE clone with the other fragmentary cDNA clones being numbered sequentially thereafter. Interestingly, in the translation of each of the four additional 5' RACE products, a tyrosine-rich region was present immediately upstream of the homeodomain that was similar in character to the IRX-2 tyrosine-rich region yet highly distinct in sequence. Also, each has a sequence similar to the hexapeptide-like motif as seen in IRX-2. As with IRX-2a, none of these cDNA fragments contained the EGF-like region found upstream of the homeodomain in araucan and caupolican. Since it is unlikely both that the level of polymorphism in the homeodomains is due to polymerase misincorporation and that alternative splicing would give rise to multiple similar yet distinct 5' ends, we postulate that these transcripts arose from distinct genes within the human genome and thus represent transcripts from five members of a large multigene family.

IRX-2 expression in Northern hybridization analysis

To investigate transcript size(s) and to detect whether expression of IRX-2 was altered in tumors relative to matched normal tissue controls, we conducted Northern blot analysis on 3 matched tissue sets (Fig 4). Under unusually stringent wash conditions, the IRX-2 IMAGE clone probe (which contains the homeobox) detected a single major 2.6 kb transcript (IRX-2a) by Northern analysis with three additional minor bands at approximately 1.8 kb, 1.6 kb, and 1.1 kb. Surprisingly, when the IRX-2(EK0.5) 5' end probe was used that did not contain the homeobox, a second major transcript was detected at about 3.9 kb (designated IRX-2b) in addition to those transcripts identified by the IMAGE probe. The IRX-2b transcript apparently lacks homeobox sequences and appears to be coordinately expressed with the IRX-2a transcript in most tissue samples examined. A similar set of transcripts both with and without the homeobox has also been observed for the mouse HoxA1 gene (LaRosa and Gudas, 1988). It is unclear, at this point, whether or not the three minor species are derived from the IRX-2 gene.

In addition to the matched tissue sets, we examined IRX-2 expression in a number of additional breast tumors. IRX-2 transcripts were detectable in all samples but were difficult to discern in some cases (Figure 4B). Surprisingly, the IDC from patient 7 clearly shows and increased level of the IRX-2b transcript relative to the IRX-2a transcript which suggests that IRX-2 is misexpressed in this tumor. The ILC from patient 21 also showed a slightly elevated level of expression of the IRX-2b transcript relative to the IRX-2a transcript. Interestingly, the FA from patient 32 clearly showed an increase in the 1.1 kb transcript relative to the two major transcripts IRX-2a and IRX-2b. Patients 7 and 21 also show this band rather prominently. Neither the 1.8 kb nor the 1.6 kb minor transcripts

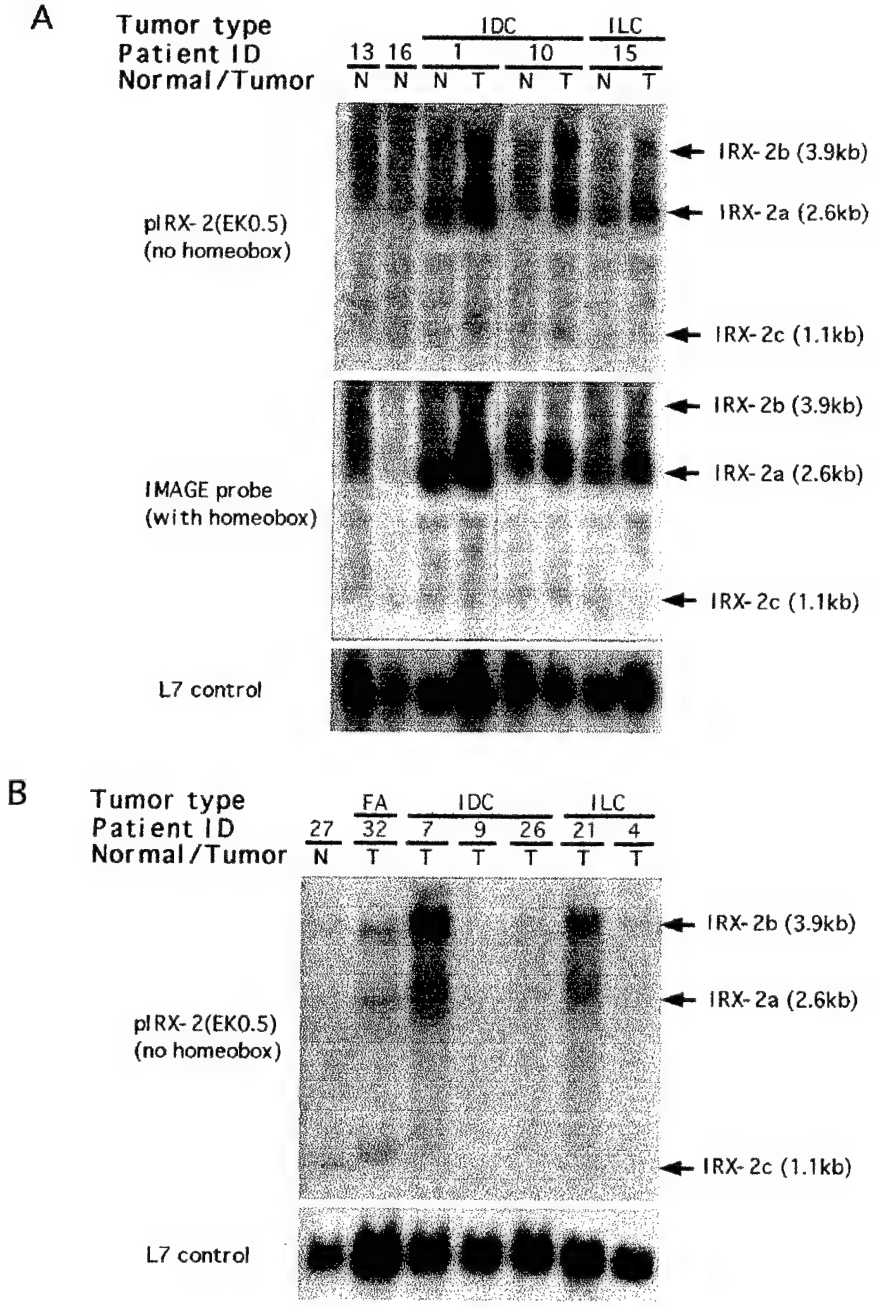

Figure 4: Expression of IRX-2 by Northern analysis. A) Matched tissue Northern blot using either the gene-specific 5' end probe pIRX-2(EK0.5) (top panel) or the IMAGE (homeobox-containing) probe (middle panel). IRX-2 transcripts are noted by arrows and size. Note that the IRX-2b transcript is not detected by the IMAGE probe. Probable nonspecific hybridization signal at 1.8 and 1.6 kb are noted by small arrows. L7 loading control hybridization is shown (bottom panel). B) Unmatched tissue Northern blot probed with pIRX-2(EK0.5) showing all three IRX-2 candidate transcripts (top panel). L7 loading control hybridizations are shown (bottom panel).

were well detected in a majority of these samples (with the possible exception of patient 21) which suggest that they are the result of non-specific hybridization of the IRX-2 probes. Given these data, we tentatively designate the 1.1 kb transcript IRX-2c. Together with the data for patient 10, these data indicate that IRX-2 is either misexpressed or overexpressed in a variety of human breast tumors.

Reliable quantitation of these results relative to the L7 loading controls was possible only with patient 10 due to gross variation in "epithelial load" between normal and tumor samples. This variation was readily observed in the tissue sections used for *in situ* hybridization. For patient 10, both normal and tumor samples were epithelium rich and thus more comparable. Both IRX-2a and IRX-2b transcripts were over-expressed 3-fold and 2.5-fold, respectively, relative to the normalized L7 loading control. The apparent over-expression seen for patient 1 is highly suggestive but complicated by the fact that the normal sample was virtually devoid of epithelium when observed in section. Though not as dramatic, the normal sample for patient 15 also showed markedly reduced epithelial content.

Finally, to determine whether IRX-2 expression was mammary specific, we also conducted Northern analysis on mRNA from adult human lung, uterus, salivary gland, and kidney using the gene specific pIRX-2(EK0.5) probe (Fig 3B). IRX-2 messages were weak but detectable in all four non-mammary tissues (data not shown) indicating that IRX-2 expression is not tissue specific.

IRX-2 transcripts are localized to mammary epithelial cells in the normal breast and in tumors.

To determine which cell types normally express IRX-2 within the mammary gland and to determine which cells express IRX-2 in various tumor types, we performed *in situ* hybridization on normal and tumor tissue from a number of patients using the gene-specific probe pIRX-2(EK0.5) derived from the 5' end of the 1.8 kb cDNA

In normal human breast tissue, IRX-2 expression is localized to mammary epithelium of small intralobular ducts (Fig 5A and 5B) and alveoli (Fig 5B and 5E) as well as in the major ducts (Fig 5D). Under high magnification, expression is almost exclusively limited to lumenal ductal (Fig 5D) and alveolar epithelium (Fig 5E). Rarely, unstained lumenal ductal epithelial cells can be observed which may represent a subclass of epithelial cells. Strikingly, expression is not observed in adjacent myoepithelial cells that line the basal surface of both ducts and alveoli nor is it observed in the periductal stroma (Figure 5D and 5E). Perhaps most interesting is the pattern of expression in ducts in apparently normal tissue adjacent to tissue affected by FA lesions (Patient 32, Fig 5F). Expression of IRX-2 transcripts is seen to be concentrated at the tips of end buds, the growing terminus of the duct, while expression levels become gradually reduced in the more mature subtending duct. This observation suggests that IRX-2 may be involved in control of growth in the immature gland.

For some patients, there appeared to be isolated cells scattered within the fatty stroma that also showed detectable IRX-2 expression, but this observation was infrequent. Sense strand control hybridization against sections containing all normal mammary epithelial cell types showed no hybridization (Figure 5C) indicating that hybridization using the antisense probe is specific.

To assess IRX-2 expression in tumors, we examined three different mammary tumor types, Infiltrating ductal carcinomas (IDC), Infiltrating lobular caricinomas (ILC), and a benign fibroadenoma (FA). IDCs presumably originate by malignant transformation

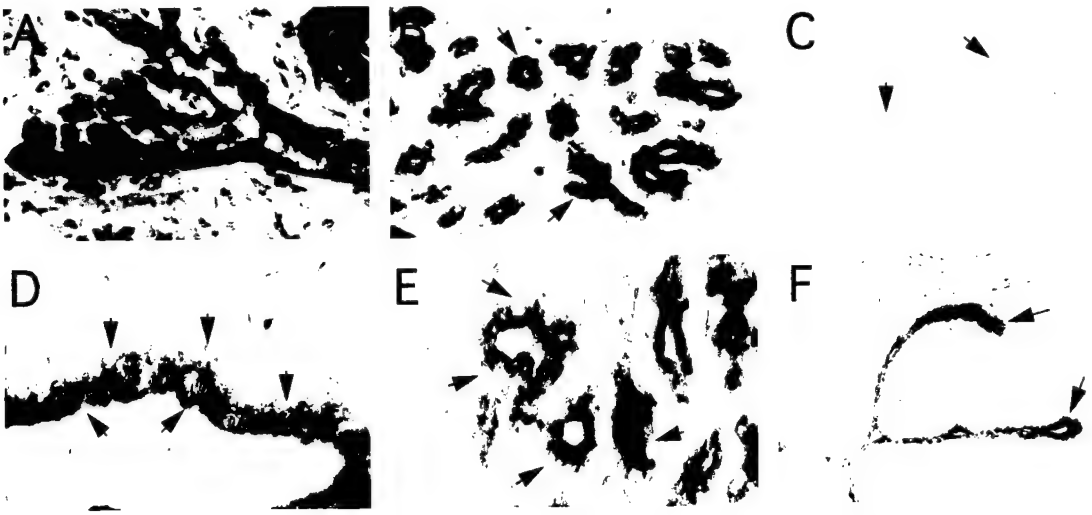

FIGURE 5: In situ expression of IRX-2 in normal tissue using thepIRX-2(EK0.5) gene-specific probe. A) Normal tissue , Patient 27. Antisenseprobe on major intralobular duct. B) Normal tissue, Patient 24. Antisenseprobe on normal lobule showing both small intralobular ducts (red arrow)and alveoli (black arrow). C) Normal tissue, Patient 24. Sense control probe on lobule (black arrows). This control panel is representative of all experiments. D) Normal tissue, patient 24. Antisense probe on a portion ofa major duct. Black arrows indicate unstained myoepithelial cells; red arrows indicate unstained lumenal epithelial cells. Not all myoepithelialcells are marked. E) Normal tissue, patient 24. Antisense probe on alveoli showing epithelial cell localization and lack of expression in myoepithelial cells (black arrows). Not all myoepithelial cells are marked. F) FA patient 32. Antisense probe on duct in a region of the tissueapparently unaffected by the FA. Black arrows indicate end bud structures.

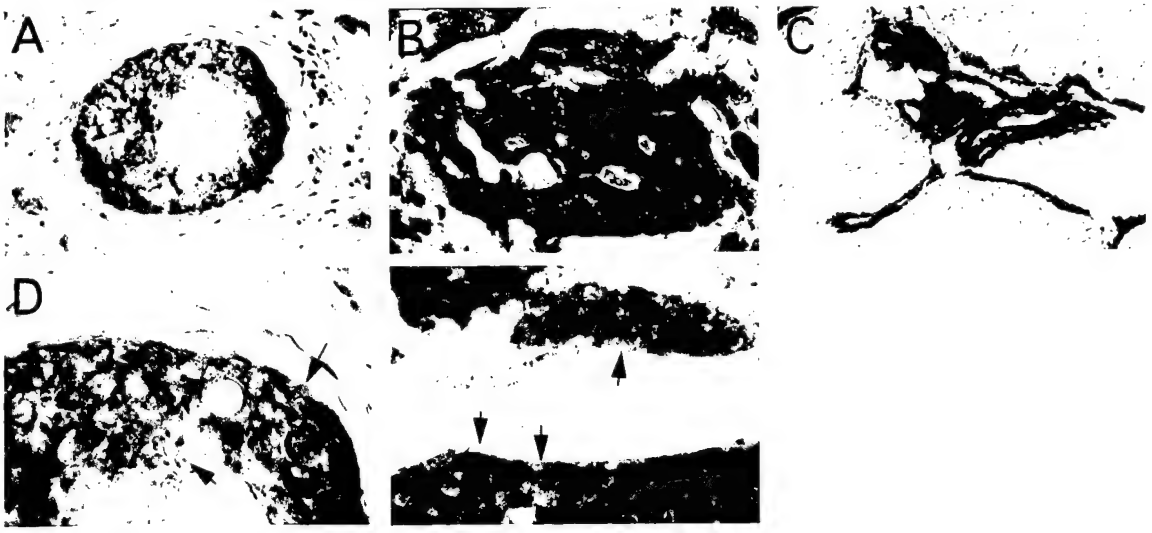

FIGURE 6: In situ expression of IRX-2 in tumors using the pIRX-2(EK0.5) gene-specific probe. A) IDC, patient 10. Antisense probe on ductal carcinoma in situ. Tumor cells are observed to nearly fill the duct. B) ILC, Patient 21. Antisense probe on lobular carcinoma in situ. An individual lobule with loss of discrete alveolar structures is shown. C)FA, Patient 32. Antisense probe on benign fibroadenoma showing a network of cells within the duct. D) High magnification. IDC, patient 10. Antisense probe showing that not all cells express IRX-2 (black arrows). The cell on the outer edge of the tumor shows position and morphology of a myoepithelial cell. E) ILC, patient 21. Antisense probe showing at least three cells that do not express IRX-2. All marked cells show position and morphology of myoepithelial cells. Sense strand control hybridizations areas shown in figure 4C.

FIGURE 7: In situ expression of IRX-related sequences using the IMAGE homeobox-containing probe. A) Normal tissue, patient 10. Antisense probe on a lobule showing epithelial cell localization of IRX-related transcripts. B) Normal tissue, patient 10. Antisense probe on major duct. Most cells do not express IRX to detectable levels. However, a limited amount of IRX-related expression can be observed in some sections. C) Normal tissue, patient 10. Sense control probe on a lobule. D) IDC, patient 10. Antisense probe showing IRX gene expression in tumor cells.

of epithelial cells within the ducts themselves. The disease progresses from ductal carcinoma *in situ,* in which tumor cells are retained within the ducts and surrounded by a basement membrane to an invasive, eventually metastatic, IDC. These are classified as grade I, II or III depending on the degree of invasiveness. Similarly ILCs are characterized by malignant cells within the lobules which eventually grow to destroy the lobule and invade the surrounding tissue, and/or migrate into the ductal system. FA, while benign, does involve hyperplastic growth and a change in duct morphology. FA is characterized by masses or networks of ductal epithelial cells that are retained within the ducts.

Figure 6A shows the expression pattern in an IDC from patient 10 that showed apparent over-expression by Northern analysis. This panel depicts IRX-2 expression in the epithelial cells that comprise the tumor. Malignant IRX-2 expressing cells of this IDC can be seen to virtually completely fill the ductal lumen. These results were representative of all IDCs examined.

IRX-2 was also expressed in the two other tumor types examined. Hybridization against an ILC from patient 21 also illustrates IRX2 expression in tumor cells (Fig 6B). Again, expression is seen in the neoplastic cells that have destroyed organotypic structure within the lobules but have not completely destroyed the lobules themselves. Again, these results were representative of all ILCs examined. IRX-2 is also expressed in the cells that contribute to fibroadenomas. Figure 6C shows a representative FA lesion in which the duct is extensively filled with a network of epithelial cells. All of the cells contributing to the lesion express IRX-2, as do the ductal epithelial cells that do not contribute to the lesion. Thus, IRX-2 is expressed in neoplastic epithelial cells of all three tumor types examined.

There are two interesting aspects of the observed expression patterns in tumors. First, high magnification examination of both the patient 10 IDC (Fig 6D) and the patient 21 ILC (Fig 6E) show that there are some cells within the tumors that do not express IRX-2, many of which show positioning and morphological features of myoepithelial cells, suggesting that this cell type is retained even in relatively advanced tumors. Second, in all three tumor types, continued IRX-2 expression does not appear to be dependent on maintenance of normal epithelial cell-cell or epithelial-stromal interactions such that, in cases where normal structures are destroyed, continued IRX-2 expression is clearly not dependent on the presence of organotypic structure. At least in terms of the IRX-2 expression phenotype, however, tumor cells still maintain epithelial cell identity.

To assess whether epithelial cell expression is a general characteristic of all closely related IRX genes, we used the homeobox-containing IRX-2 IMAGE clone probe for *in situ* hybridization against tissue sections from many of the same patients used with the gene-specific probe. We had expected that some IRX genes would show epithelial expression and some IRX genes would show stromal expression resulting in hybridization signal over the entire gland. Surprisingly, we observed very strong hybridization primarily in normal lobule-alveolar structures (Fig. 7A) and very little expression in normal ducts (Fig. 7B), with no detectable expression in periductal or fat pad stroma. Since we know IRX-2 is expressed in both lobules and ducts using the pIRX-2(EK0.5) probe, this observation implies that at least one closely related IRX gene is more highly expressed in lobules than in ducts and thus is, under this set of conditions, detectable using conditions that do not produce an IRX-2 signal. By contrast, tumor cells of IDCs (presumably duct derived) show dramatic expression of IRX genes as evidenced by specific signal in tumor cells within the ducts in patient 10 (Figure 7D). Since we observed very little hybridization in epithelium of normal ducts using the IMAGE probe, this observation implies that at least one IRX gene is overexpressed in this tumor. Coupled with the Northern hybridization analysis, IRX-2 is a likely candidate for this overexpressed gene.

Objective 3. *Are homeobox genes regulated by mammogenic hormones or growth factors? By retinoic acid?*

The breast is the quintessential example of an endocrine target organ, in which each developmental stage, from early budding, through growth and branching of the ductal tree, milk production, and finally involution, is regulated by a complex and continually changing hormonal milieu. It is therefore reasonable to suppose that genes regulating growth, patterning, and function will be under regulation of one or more of the mammogenic hormones. Indeed, if regulatory gene expression were unaffected by levels of reproductive hormones, one would be doubtful that their expression was interestingly linked to mammary development or function.

In the 1995 progress report we reported in detail evidence supporting a role for estrogen in regulating Msx-2. Although these results were confirmed and expanded, the conclusions remain the same, and the reader is referred to the attached reprint (Appendix) for details. The main findings are:

1. Msx-2 gene expression was reducedin glands from ovariectomized mice.
2. Levels of Msx-2 returned to normal when ovariectomized mice were treated with estrogen.
3. In endocrine-intact animals, antiestrogens administered directly to the mammary glands using slow-release plastic pellets reduced the levels of Msx-2 transcripts, indicating that endogenous estrogen is required for normal steady-state transcript levels.

Objective 4. *Does misexpression of homeobox genes in situ influence the incidence of precancerous and cancerous lesions?*

Functional analysis of expressed homeogenes is being carried out by overexpressing homeogenes in mouse mammary gland and examining the resulting phenotype *in situ.* Preneoplastic and neoplastic changes, as well as alterations to normal growth patterns will be related to expression. In the case of the mouse mammary gland a unique variation on these techniques is available. Mammary epithelium cultivated as a monolayer can be transfected *in vitro,* where selection can be used to enrich the cultures for cells carrying introduced genes for antibiotic resistance. These cells are injected into a gland-free fat pad where they are capable of regenerating mammary gland. Even small variations in growth rate, pattern, or functional activity of these regenerated glands can be readily recognized by experienced observers. Areas of interest can be selected and transplanted into other fat pads, creating tissue lines of genetic variants that can be tested for incidence of preneoplastic and neoplastic changes.

We have begun these experiments. Preliminary data show that mammary monolayer cultures can be transplanted into the gland-free fat pads of isogenic mice with a 80-90% success. Full length transcripts of both Msx-2 and Hoxa-1 have been incorporated into retroviral vectors, propagated in producer cells, and the harvested viruses used to infect mammary epithelial cells in culture. These cells have been implanted into hosts, and in a few weeks we will have our first chimeric transgenic mammary glands. In the next progress report the experimental details will be given, and we hope to have interesting data to report.

CONCLUSIONS

Quite aside from any role that IRX homeogenes may play in the development of the breast or of breast cancer, the discovery of a large, new, highly conserved class of human homeobox-containing genes is a significant event. In all cases where the functional roles of other homeogenes have been elucidated, mainly through targeted gene inactivation, they have been shown to play major roles in regulating pattern formation in the embryo. There is no reason to suppose that IRX genes will be different.

In the breast, the high levels of expression and the highly restricted pattern of expression in certain but not all mammary epithelial cells speaks to a functional role in breast morphogenesis or functional differentiation. With respect to breast cancer, IRX is interesting in showing a quite different pattern of expression than that seen with other homeotic genes, which typically are underexpressed in malignancies (with the exception of certain Hoxa genes). It also may be significant that IRX-2 is overexpressed in some cancers, and that relative transcript levels are altered within several different tumor samples of different tumor types. Together, these data present the possibility that IRX-2 may play a role in cell proliferation and/or tumor progression

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APPENDIX

Attached Reprint:

Friedmann, Y., and Daniel, C.W. (1996) Regulated expression of homeobox genes Msx-1 and Msx-2 in mouse mammary gland development suggests a role in hormone action and epithelial-stromal interactions. Dev. Biol. 177, 347-355

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PERSONNEL ON SALARY

Charles W. Daniel, P.I.
Michael Lewis, Postdoctoral Fellow
Phyllis Strickland, Staff Research Assoc
Sarajane Ross, Graduate Student
Charles Slugnet, Staff Research Associate

DEVELOPMENTAL BIOLOGY 177, 347–355 (1996)
ARTICLE NO. 0168

Regulated Expression of Homeobox Genes *Msx-1* and *Msx-2* in Mouse Mammary Gland Development Suggests a Role in Hormone Action and Epithelial–Stromal Interactions

Yael Friedmann[1] and Charles W. Daniel

Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, Santa Cruz, California 95064

The murine homeobox genes *Msx-1* and *Msx-2* are related to the *Drosophila msh* gene and are expressed in a variety of tissues during mouse embryogenesis. We now report the developmentally regulated expression of *Msx-1* and *Msx-2* in the mouse mammary gland and show that their expression patterns point toward significant functional roles. *Msx-1* and *Msx-2* transcripts were present in glands of virgin mice and in glands of mice in early pregnancy, but transcripts decreased dramatically during late pregnancy. Low levels of *Msx-1* transcripts were detected in glands from lactating animals and during the first days of involution, whereas *Msx-2* expression was not detected during lactation or early involution. Expression of both genes increased gradually as involution progressed. *Msx-2* but not *Msx-1* expression was decreased following ovariectomy or following exposure to anti-estrogen implanted directly into the gland. Hormonal regulation of *Msx-2* expression was confirmed when transcripts returned to normal levels after estrogen was administered to ovariectomized animals. *In situ* molecular hybridization for *Msx-1* showed transcripts localized to the mammary epithelium, whereas *Msx-2* expression was confined to the periductal stroma. Mammary stroma from which mammary epithelium had been removed did not transcribe detectable amounts of *Msx-2*, showing that expression is regulated by contiguous mammary epithelium, and indicating a role for these homeobox genes in mesenchymal–epithelial interactions during mammary development. © 1996 Academic Press, Inc.

INTRODUCTION

Mammary development and function are driven by a complex network of hormones acting systemically, which in turn influence peptide growth factors that regulate developmental events at the tissue level (Topper and Freeman, 1980; Dembinski and Shiu, 1987). Estrogen and progesterone are particularly crucial signals in growth and morphogenesis of the breast. It is likely that these hormones control regulatory genes that serve to coordinate developmental interactions and to specify pathway decisions in the developing gland. Because homeobox genes function as master regulators of embryonic events in a variety of organisms including the mouse (Morgan *et al.*, 1992; Balling *et al.*, 1989; Wolgemuth *et al.*, 1989; Ramirez-Solis *et al.*, 1993; Le Mouellic *et al.*, 1992), and because the expression of several *Hox* genes was recently reported in the mammary gland (Friedmann *et al.*, 1994), the mammary gland is a candidate target for hormone-regulated homeobox gene action.

In the mammary gland, genes regulating morphogenesis and growth are likely to be associated with epithelial–stromal interactions. With the onset of ovarian function at 3–4 weeks of age, the mouse mammary ductal system enters a phase of rapid growth and morphogenesis, in which continuing inductive interactions between mammary epithelium and contiguous stroma result in growth and patterning of a ductal tree that fills the adipose-rich stroma (Sakakura *et al.*, 1979). The ductal epithelial cells may follow one of several differentiative pathways depending on their position within the growth buds at the tips of the ductal branches, becoming cells for ductal walls, milk synthesis and secretion, or contraction (Williams and Daniel, 1983). In concert with this epithelial differentiation, the stroma adjacent to the growing duct becomes rich in fibrocytes, producers of

[1] Present address: Department of Oncology, P.O. Box 12000, Hadassah University Hospital, Jerusalem 91120, Israel.

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materials of the fibrous extracellular matrix which encases mammary ducts. (Williams and Daniel, 1983).

The homeobox is a relatively conserved 183-nucleotide sequence encoding a DNA-binding domain found in many genes playing key roles in *Drosophila* embryogenesis. These genes are classified according to their homeobox sequence and chromosomal location. Thirty-eight mammalian homeobox-containing genes, the *Hox* genes, are found in clusters on four chromosomes. Gene disruption and gain-of-function mutations generated in mice have shown that improper expression of *Hox* genes leads to developmental defects (Chisaka and Capecchi, 1991; Kessel and Gruss, 1991; Lufkin *et al.*, 1991), many of which represent homeotic transformations (Morgan *et al.*, 1992; Ramirez-Solis *et al.*, 1993; Small and Potter, 1993; Le Mouellic *et al.*, 1992).

Other mammalian homeobox genes are not located within these clusters and form smaller classes based on sequences that relate them to other conserved motifs such as *Pax* and *Oct* (Herr *et al.*, 1988; Epstein *et al.*, 1991; Hill *et al.*, 1991; Palmieri *et al.*, 1994; Corcoran *et al.*, 1993). The *Drosophila melanogaster* muscle segment homeobox (*msh*) gene contains a homeobox which is markedly divergent from that of any other characterized *Drosophila* genes. In *Drosophila*, *msh* is mainly expressed in the central nervous system and in segmented striated muscles of the body wall. In the mouse there appear to be three distinct msh-like genes, named *Msx-1*, *Msx-2*, and *Msx-3*, which are found at separate loci and are not clustered (Hill *et al.*, 1989; Robert *et al.*, 1989; Monaghan *et al.*, 1991; Holland, 1991). Closely related versions of *Msx-1* and *Msx-2* have been identified in a variety of vertebrate species including zebrafish (Ekker *et al.*, 1992), *Xenopus* (Su *et al.*, 1991), and the chick (Coelho *et al.*, 1991).

Msx-1 and *Msx-2* show a closely associated, interactive pattern of expression throughout early embryonic development (MacKenzie *et al.*, 1991a, 1991b; Monaghan *et al.*, 1991). The earliest expression of both genes is detectable in primitive streak mesoderm, followed by expression in neural crest cells and their derivatives. Later expression patterns have been examined by *in situ* hybridization methods in the development of several organs, including the mouse and chick limb bud (Nohno *et al.*, 1992; Davidson *et al.*, 1991; Robert *et al.*, 1991), mouse tooth bud (Mackenzie *et al.*, 1991a, 1992; Jowett *et al.*, 1993), chick heart (Chan-Thomas *et al.*, 1993), and chick craniofacial development (Nishikawa *et al.*, 1994). The results suggested that the two genes play a role in epithelial–mesenchymal interactions in these developing organs.

In a previous paper we reported expression in the mammary gland of several genes from the four *Hox* clusters and the altered expression of some during tumorigenesis (Friedmann *et al.*, 1994). In this paper we describe the expression of *Msx-1* and *Msx-2* RNA in different stages of mammary development. To move closer to a functional analysis, we have examined the effects of experimentally altering tissue interactions and manipulating mammogenic steroids.

METHODS

Animals. C57BL/crl mice were used for collection of the inguinal mammary glands in all RNA preparations. Virgin mice were chosen randomly from multiple cages to minimize the chances of selecting animals in a particular stage of estrus. For involuting glands, pups were weaned 10 days after birth and the following day was counted as Day 1 of involution. Thoracic glands of virgin and pregnant C57BL/crl mice were used for *in situ* hybridization.

Surgery. Ovariectomy was carried out at about 5 weeks of age (animal weight 16–17 g) and glands were collected 4 weeks later to allow complete mammary regression. When estrogen implants were used, they were implanted subcutaneously 6 weeks after ovariectomy and glands were collected 4 days later. This staging was necessary because after estrogen replacement therapy, the mammary glands from ovariectomized animals more closely resemble developing glands in younger 6-week animals.

The inguinal fat pads were cleared of epithelium at 3 weeks of age by removing the portion of the gland containing epithelium and cauterizing the nipple area and associated blood vessels (DeOme *et al.*, 1959).

Implants. EVAc (Elvax 40P) was a gift from DuPont Chemical Co. (Universal City, CA). Anti-estrogen ICI 164,384 was a gift from ICI Pharmaceutical (Cheshire, England). 17 β-Estradiol is from Sigma (E-8875). Implant preparation is described in detail elsewhere (Silberstein and Daniel, 1982). Briefly, anti-estrogen was dispersed in 0.125 ml of EVAc that had been dissolved in dichloromethane (20% w/v). This mixture was quick-frozen and evaporated under vacuum, and the polymer matrix with entrapped chemical was then cut to form pellets containing 250 μg anti-estrogen and surgically implanted (typical implant weight, 0.5 mg). 17 β-Estradiol was mixed with dichloromethane and serially diluted to a final dose of 50 ng implants per animal. Recipient mice were anesthetized with an interperitonial injection of Nembutal (60 $\mu g\ g^{-1}$ body wt) and the number 3 mammary glands (in the case of anti-estrogen) were exposed by reflecting the skin from a midline ventral incision. A small pocket was made in the mammary fat pad using Dumont forceps, which were then used to insert the implant. Estrogen implants were inserted through a small incision subdermally at the back of the neck. The skin was then closed with wound clips and the animals were allowed to recover in an atmosphere of 95% O_2/5% CO_2.

RNA preparation and Northern hybridization. Inguinal mammary glands were frozen in liquid nitrogen immediately after removal, and total RNA was prepared by the guanidine isothiocynate (4 *M*), cesium chloride (5.7 *M*) method (Ausubel *et al.*, 1989). Total RNA from the glands (the number and age of animals used for each experiment are given in the figure legends) was isolated. In several cases poly(A)$^+$ RNA was purified by oligo(dt)–cellulose chromatography as described (Sambrook *et al.*, 1989). Five micrograms of poly(A)$^+$ enriched RNA, or 25 μg total RNA was electrophoresed in 1.0% agarose containing 2.3 *M* formaldehyde in Mops buffer (0.2 *M* morpholinopropane sulfonic acid, 50 m*M* sodium acetate, 5.0 m*M* EDTA, pH 7.0). RNA was transferred to a nylon transfer membrane (Magna NT, Micron Separation Inc., 0.45 μm) by the established procedure of Maniatis *et al.* (1982). Northern hybridizations were carried out under high stringency conditions, using ^{32}P-random-primed labeled (1×10^9 to 10×10^9 counts minute^{-1} μg^{-1}) murine *Msx-2* and *Msx-1* and human L7 cDNAs. The *Msx-2* fragment is ~400 base pairs (bp), derived from the 3′ end of the gene. It does not include the homeobox but does include ~200 bp of the 3′ UTR. The *Msx-1* fragment is ~850 bp, derived from the 3′ end

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of the gene. It begins at the C-terminal half of the homeobox and includes ~550 bp of the 3′ UTR. Washes after hybridizations were in 0.1× SSPE/0.1% sodium dodecyl sulfate at 65°C. All Northern hybridizations were repeated at least twice using different blots.

In situ hybridization. Mammary glands from virgin and pregnant animals were fixed for 3 hr in 4% paraformaldehyde/PBS, dehydrated through a graded series of ethanols to xylene, and embedded in paraffin wax. Seven-micrometer sections were cut, floated on slides coated with 3-aminopropyltriethoxysilane (Sigma), and baked onto slides overnight on a slide warmer at 45°C. Sections were dewaxed through two changes of xylene and rehydrated through graded series of ethanols. Sections were then digested with proteinase K (1 μg ml^{-1} in 10 m*M* Tris–HCl, 5 m*M* EDTA, pH 7.5) at 37°C for 30 min and the reaction was stopped with two changes of H_2O and one wash in PBS for 2 min.

To avoid nonspecific binding of RNA probes, slides were prehybridized for 1 hr at 45°C with hybridization buffer (see below) that did not include the RNA probe. Slides were hybridized at 45°C for 16 hr under siliconized coverslips in a solution containing 50% formamide, 3 m*M* NaCl, 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 150 μg ml^{-1} tRNA, 1 mg ml^{-1} yeast total RNA, 10% dextran sulfate, 1% blocking solution (blocking reagent for nucleic acid hybridization, Boehringer-Mannheim Genius system kit), and 800 ng ml^{-1} digoxigenin labeled RNA probe. After hybridization, coverslips were removed in 2× SSPE and then slides were rinsed twice, for 1 hr each time in 0.2× SSPE at 50°C.

To avoid nonspecific binding of anti-digoxigenin antibody, slides were treated for 45 min at room temperature with 2% blocking solution in 100 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl and then for 45 minutes in BSA wash solution [1% BSA (Sigma A-7030), 0.3% Triton X-100, 100 m*M* Tris–HCl, pH 7.5, and 150 m*M* NaCl] at room temperature. Slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody in 2% blocking solution under siliconzed coverslips for 16 hr at room temperature. Coverslips were removed in BSA wash solution, followed by two more BSA washes, and then incubated in 2% blocking solution for 30 min followed by 2 min in a solution containing 100 m*M* Tris–HCl, pH 9.5, 100 m*M* EDTA, and 50 m*M* $MgCl_2$. To visualize probes, slides were incubated with a pair of colorimetric substances, NBT and x-phosphate, in the above solution (as described in Boehringer-Mannheim The Genius System User's Guide for Filter Hybridization, Version 2.0) for various times ranging from 5.5 to 24 hr. When the desired intensity was reached, the reaction was stopped in 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.5. Slides were dehydrated through graded alcohols into xylene and coverslipped.

Riboprobes were labeled with digoxigenin labeling mix (NTP labeling mixture 10×, Boehringer-Mannheim Catalog No. 1277 073), using the appropriate SP6 or T7 transcription system. All *in situ* hybridizations were repeated at least four times.

RESULTS

Expression of Msx-1 and Msx-2 Transcripts Levels during Mammary Gland Development

The expression level of *Msx-1* and *Msx-2* transcripts in various stages of mammary development was evaluated by Northern blot hybridization to poly(A)$^+$ enriched RNA isolated from mouse mammary glands at several stages of development. The Northern blot was hybridized consecutively with probes for *Msx-1*, *Msx-2*, and L7 (as a control for the amount of RNA and its integrity). *Msx-1* (~2050 bp) and *Msx-2* transcripts (~1300 and ~2300 bp) were present in glands from virgin animals and glands from animals during early pregnancy (5–8 days post coitus) (Fig. 1). Transcript levels of both genes decreased substantially in glands from animals in late stages of pregnancy (15–18 days post coitus). In lactating glands *Msx-2* expression was not detected, while *Msx-1* transcripts were seen at low levels. The transcript size of *Msx-1* in glands from lactating animals was somewhat smaller (~1.9 kb) than the transcript size from other stages of the mammary cycle.

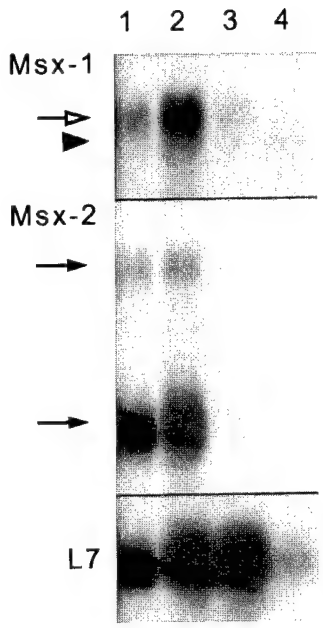

FIG. 1. RNA expression of *Msx-1* and *Msx-2* during stages of mouse mammary gland development. Lane 1: 25 immature virgin mice were taken; Lane 2: 7 mice were taken 5–8 days into timed pregnancies; Lane 3: five 15- to 18-days pregnant mice were taken; Lane 4: 3 animals were taken 3–4 days into lactation. Each lane contains 5 μg of poly(A)$^+$ RNA. L7 mRNA was used as a loading control. Open arrow points to *Msx-1* transcripts, solid arrows point to *Msx-2* transcripts. Arrowhead points to smaller *Msx-1* transcript observed only in lactating glands.

The final stage of the mammary cycle is involution, in which, following weaning, secretory tissue is destroyed by apoptotsis as the gland reorganizes to a form resembling its prepregnancy state. Total RNA was extracted from glands of mice that were lactating for 10 days before pups were weaned, and their glands were removed at several time points during involution. Figure 2 shows the expression of *Msx-1* and *Msx-2* during involution. *Msx-1* was expressed at low levels in the first 3 days of involution (lanes 2 and 3). In the fourth day of involution expression increased and remained at similar levels thereafter. *Msx-1* transcript size in glands that were involuting for 2 days was the same as in lactation. In the third day after weaning both transcripts

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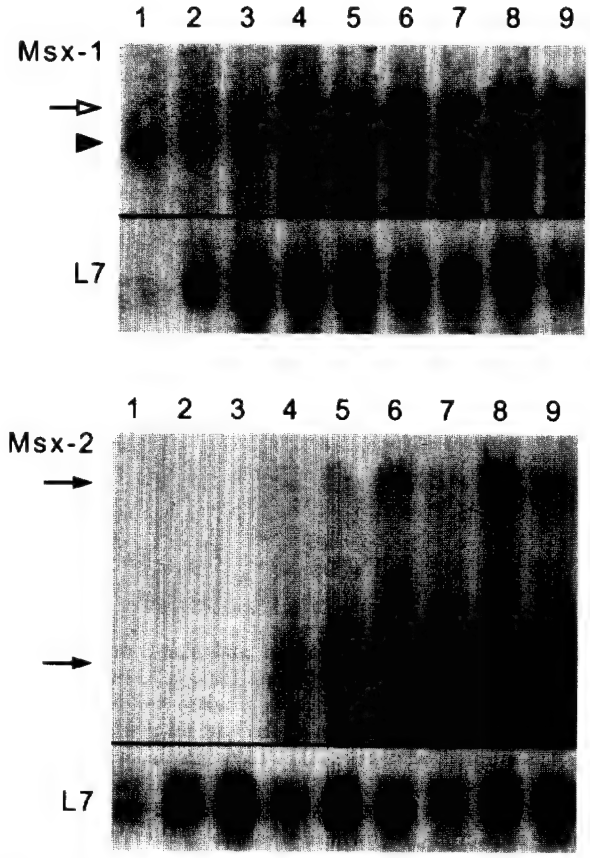

FIG. 2. Northern analysis of *Msx-1* and *Msx-2* in involuting mammary glands. Mice were lactating for 10 days before pups were removed. The next day is considered the first day of involution. RNA was extracted from glands involuting for different numbers of days. The Northern analysis was performed as described under Materials and Methods. Lane 1: lactating glands. Lane 2: 2 days involuting. Lane 3: 3 days involuting. Lane 4: 4 days involuting. Lane 5: 6 days involuting. Lane 6: 8 days involuting. Lane 7: 10 days involuting. Lane 8: 12 days involuting. Lane 9: 14 days involuting. Each lane contains 20 μg total RNA. Lanes 1–3: three animals were taken. Lanes 4–5: five animals were taken. Lanes 6–9: six animals were taken. Solid arrows point to *Msx-2* transcripts. Open arrow points to *Msx-1* transcript. Arrowhead points to *Msx-1* smaller transcript in lactating and early involuting glands.

were visible, after which the larger transcript size, that which was detected in other stages of gland development, was the predominant one. *Msx-2* transcripts could not be detected in the first 3 days of involution. Expression increased gradually in Days 4–8 and then reached a plateau.

Msx-2 Expression in Glands from Ovariectomized Animals and after Estrogen Replacement

To determine if *Msx-1* and *Msx-2* transcript levels are regulated by ovarian secretions, we isolated RNA and made a Northern blot from glands of animals that had been ovariectomized at two time points during the gland development. Ovariectomy was performed either at the age of 5 weeks, when the mouse initiates estrus cycles (puberty), or at the age of 12 weeks when the mouse is already mature and cycling (adulthood). After ovariectomy, mice were allowed to recuperate for 4 weeks, a time period that was determined adequate for ovarian steroids to be depleted from the tissues, when glands were taken.

The expression level of *Msx-1* in glands from ovariectomized mice was similar to that in glands from intact controls (Fig. 3). On the other hand, the expression of *Msx-2* was lower in glands from ovariectomized animals at both time points compared to glands from intact controls (Fig. 3), suggesting that *Msx-2* RNA level is up-regulated by ovarian secretions.

To further test this hypothesis, *Msx-2* was hybridized to mammary gland RNA from animals that were ovariectomized at 5 weeks of age, and in which estrogen was restored by subcutaneous implants at 11 weeks of age. Glands were collected 4 days after estrogen was implanted. When estrogen was replaced in animals that were ovariectomized, *Msx-2* levels (Fig. 4, lane 2) returned to levels found in glands from intact animals (Fig. 4, lane 3).

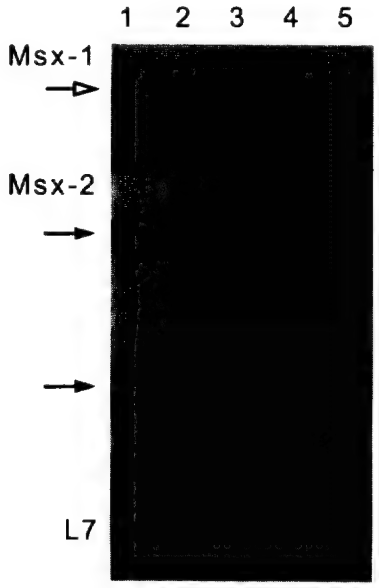

FIG. 3. RNA expression of *Msx-1* and *Msx-2* in the mammary gland in response to ovariectomy. Poly[A]$^+$ RNAs were extracted from glands of twenty 5-week-old endocrine intact mice (lane 1); fifteen 8- to 9-week-old endocrine-intact mice (lane 2); thirty 9-week-old mice that were ovariectomized at 5 weeks of age (lane 3); fifteen 16-week-old endocrine-intact mice (lane 4); twenty-five 16-week-old mice that were ovariectomized at 12 weeks of age (lane 5). Northern analysis was performed as described under Materials and Methods. Each lane contains 5 μg of poly[A]$^+$ RNA. Solid arrows point to *Msx-2* transcripts. Open arrow points to *Msx-1* transcript.

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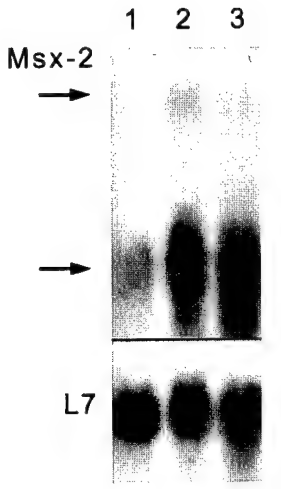

FIG. 4. Expression of *Msx-2* RNA in the mammary gland in response to estrogen replacement in ovariectomized mice. RNA was extracted from mammary glands of mice that were ovariectomized at the age of 5 weeks, let mature for 6 more weeks, when estrogen was added subcutaneously. Glands were collected 4 days later (lane 2). RNA levels are compared to RNA from glands of 6 weeks endocrine-intact animals (lane 3) and to glands from mice that were ovariectomized at 5 weeks of age and let mature for an additional 6 weeks prior to glands collection (lane 1). For each lane 10 animals were taken. Northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

Expression of *Msx-2* RNA was also examined in glands that were treated with pure anti-estrogen in endocrine-intact animals. The pure anti-estrogens are estrogen antagonists without the estrogenic properties that are, paradoxically, associated with many conventional anti-estrogens. Pure anti-estrogens have been shown to have a highly localized inhibitory effect on mammary epithelial growth and morphogenesis when tested in a natural endocrinological and physiological milieu (Silberstein *et al.*, 1994). EVAc implants, containing the pure antiestrogen ICI 164,384, were implanted directly into the mammary gland in a concentration that was shown to influence only the implanted gland, leaving other glands in the same animal unaffected (Silberstein *et al.*, 1994). RNA was extracted from glands treated with anti-estrogen and from untreated contralateral glands. Glands that were treated with anti-estrogen show reduced levels of *Msx-2* RNA compared to the untreated glands (Fig. 5). These results support the hypothesis that *Msx-2* RNA is up-regulated by ovarian secretions, of which estrogen is a component.

Spatial Localization of Msx-1 and Msx-2 RNA in the Mammary Gland

In situ hybridization was performed on sections of mammary gland tissue using gene-specific probes. Figure 6A shows *Msx-2* RNA localized to the periductal stromal cells, where these cells are forming extracellular matrix (ECM) in coordination with ductal growth. ECM is maintained around the ducts in the quiescent gland of the nonpregnant mouse. In glands from pregnant mice *Msx-2* is localized to stromal cells adjacent to mammary ducts (Fig. 6B) and in most cases not around the developing secretory alveoli or away from gland.

To further test the involvement of *Msx-2* in epithelium–stroma interactions, we examined mammary gland-free fat pads from which the epithelial component had been surgically ablated in prepubertal mice (DeOme *et al.*, 1959). As adults these mice carry inguinal glands consisting solely of mammary adipose stroma which is devoid of any mammary epithelial component. RNA extracted from gland-free fat pads was probed with *Msx-2* and no transcripts were detected, even after a long exposure time (Fig. 7). *In situ* hybridization did not show any detectable *Msx-2* messages either (not shown). This indicates the essential role of epithelium in inducing mesenchymal expression of *Msx-2*.

Figure 6C shows *Msx-1* transcripts localized to the epithelium in glands from pregnant mice. Epithelial localization was found in glands from virgin mice as well (not shown).

DISCUSSION

In a previous paper (Friedmann *et al.*, 1994), we described the expression of *Hox* genes in mouse mammary gland development, in precancerous lesions, and in malignancy. Here we extend these experiments to include developmental studies on expression of two homeobox-containing

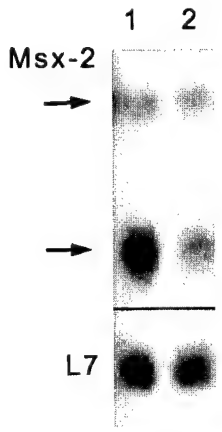

FIG. 5. RNA expression of *Msx-2* in the mammary gland in response to implanted anti-estrogen. RNA was extracted from glands of five pubescent mice that were implanted with 100 μg/gland of anti-estrogen ICI 164,384 for 4 days (lane 2) or from untreated contralateral glands (lane 1). Northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

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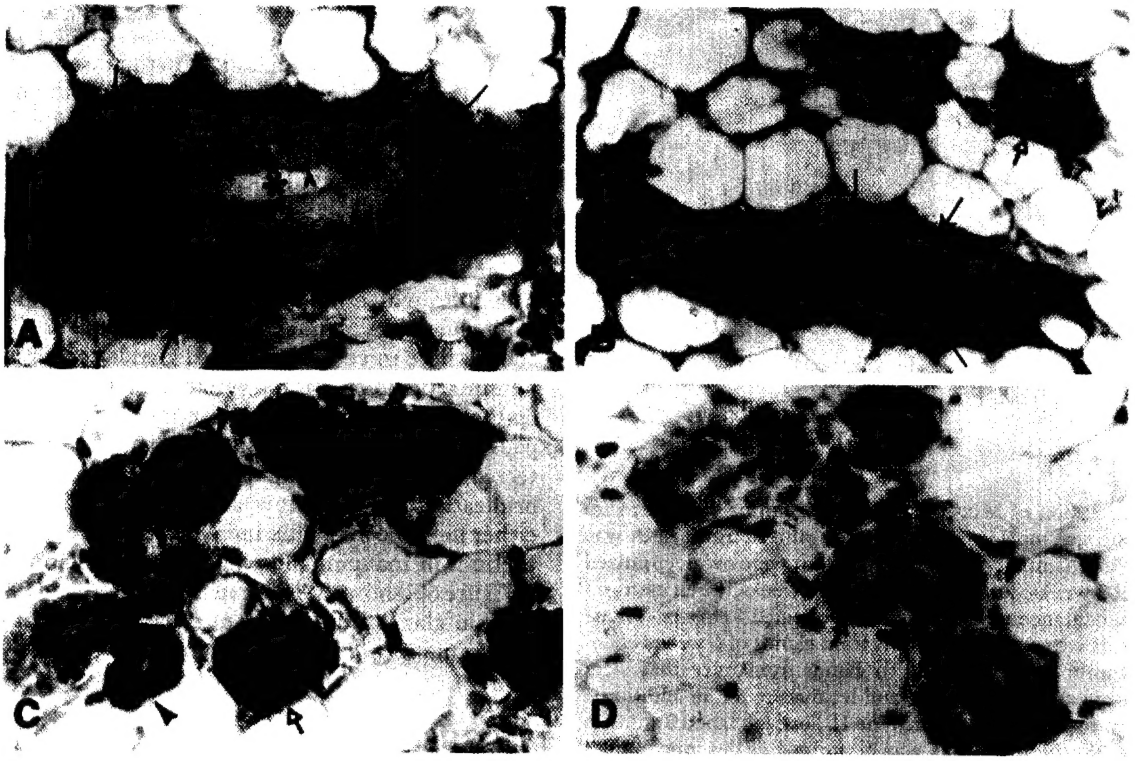

FIG. 6. Spatial expression of *Msx-1* and *Msx-2* mRNA in mammary gland. Riboprobes were labeled with DIG-11-UTP. Fragments labeled were the same as described for Northern blot hybridization (see Materials and Methods). (A) *Msx-2* expression in a gland of mature, virgin mouse. (B) *Msx-2* expression in a gland of a pregnant mouse. (C) *Msx-1* expression in mammary gland of a pregnant mouse. (D) *Msx-1* sense control probe in a gland of a pregnant mouse. It is representative of the controls for other stages of gland development for *Msx-1* and *Msx-2* sense probes. Solid arrowheads point to *Msx-1*-positive epithelial cells. ⟩ points to *Msx-2*-negative epithelial cells. Solid arrows point to *Msx-2*-positive periductal stromal cells. Open arrows point to lobule-alveolar structures in pregnant glands. Asterisks indicate lumens. Bar, 15 μm.

genes, *Msx-1* and *Msx-2*, which are located on chromosomes 5 (Hill *et al.*, 1989) and 13 (Bell *et al.*, 1993), respectively, and are not linked to other known homeogenes. *Msx-1* and *Msx-2* RNAs were expressed during mammary gland development in a stage-dependent manner, appearing in the virgin animal, declining during pregnancy and lactation, and increasing again at the later stages of involution. The expression patterns and the different expression levels of the two genes during the various stages of the gland development indicate differential regulation of *Msx-1* and *Msx-2*, as well as developmental regulation of their expression in the mammary cycle. Absence of detectable expression of *Msx-2* and low levels of expression of *Msx-1* during lactation may be due in part to high levels of milk protein transcripts that may dilute other mRNAs, as seen by the L7 loading control. The smaller size of *Msx-1* transcripts from lactating glands may indicate that an alternative protein with a possible different role is produced.

Increasing levels of expression of both *Msx-1* and *Msx-2* as involution progresses may indicate that both genes participate in the later stages of glandular reorganization, rather than being required for earlier apoptotic events. Though *Msx-1* is expressed from the beginning of involution, the expression level is low and the transcript size in the second day after weaning is the same as in the lactating gland. This indicates that the detected RNAs were probably leftover from lactation, and they may not participate significantly in the early stages of involution. *Msx-1* synthesis *de novo* appears to start only after 4 days postweaning, supporting a role for this gene in the later stages of involution. Both *Msx-1* and *Msx-2* transcripts showed a relatively high degree of degradation during involution on Northern blots. Because there is extensive tissue rearrangement during involution, this degradation is probably part of this process and may be specific to certain classes of transcripts, as the L7 control does not show the same degree of degradation.

The mammary gland is an endocrine target organ of considerable complexity. The ovarian steroids, estrogen and progesterone, are critically involved in the stimulation of mammary growth at puberty and during pregnancy (Lyons, 1958; Nandi, 1958), and genes that are involved in regulating tissue-specific responses to these hormones are likely to

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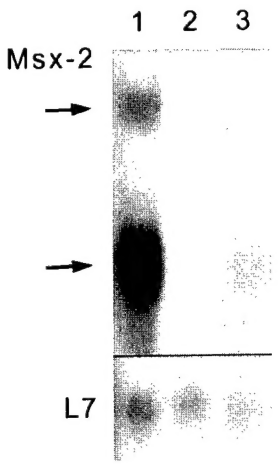

FIG. 7. RNA expression of *Msx-2* in gland-free fat pad. RNA was extracted from glands whose epithelial component had been removed in the 3-week-old mice. Glands were collected 2 months after surgery (lane 2). Expression is compared to levels in glands of epithelium-intact mature virgin mice (lane 1) and to glands of ovariectomized mice (lane 3). For lanes 1 and 3, 5 animals were taken; for lane 2, 15 animals were taken. The northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

be directly or indirectly influenced by levels of circulating steroids. If *Msx-1* or *Msx-2* are involved in the development of the mammary gland, their activity is expected to be linked to mammogenic endocrine secretions, however indirectly. To test this, glands from ovariectomized animals were examined for the expression of *Msx-1* and *Msx-2* RNA. *Msx-1* transcript levels did not change conspicuously in response to ovariectomy. On the other hand, *Msx-2* transcripts levels decreased in glands from animals that were ovariectomized either at puberty or at adulthood relative to glands taken from same age intact animals, suggesting that ovarian secretions up-regulate *Msx-2* expression. When estrogen was replaced in ovariectomized mice, *Msx-2* levels returned to levels similar to those seen in glands in endocrine-intact mice.

This indicates that estrogen has the potential to regulate *Msx-2* expression, but the question of whether physiological, circulating levels of endogenous estrogen could do so remained unanswered. *Msx-2* expression was then studied in glands that were treated *in situ* with anti-estrogen. As with ovariectomized animals, transcripts levels declined, but in this case only the treated glands were affected, while untreated contralateral glands displayed normal levels of *Msx-2* expression. Because the anti-estrogen used belongs to a class of agents that lacks estrogenic activity (Wakeling and Bowler, 1988; Wakeling *et al.*, 1991), this experiment demonstrates that estrogen is required for and normally functions as a regulator of *Msx-2* expression. More work should be done to determine if the effect is direct or through downstream mediators.

Msx-1 and *Msx-2* belong to a family of genes which are related to the *Drosophila Msh* genes. Although a precise function has yet to be established for any of the *msh*-related genes, their spatial domains of expression, as well as features of their regulation, suggest that they are key participants in basic developmental processes. Msx-1 and Msx-2 are involved in epithelial–mesenchymal interactions in developing organs, including the mouse and chick limb buds (Davidson *et al.*, 1991; Robert *et al.*, 1991), mouse tooth development (Jowett *et al.*, 1993; Satokata and Maas, 1994), and mouse and human craniofacial bone development (Satokata and Maas, 1994; Liu *et al.*, 1994, 1995; Jabs *et al.*, 1993). Epithelium–mesenchyme interactions are crucial to the development of the mammary gland (Sakakura *et al.*, 1976; Sakakura, 1987), and localization of *Msx-1* and *Msx-2* transcripts by *in situ* hybridization was expected to provide insights into whether these genes play a morphogenetic role in mammary development.

In glands from virgin and pregnant animals, *Msx-1* was localized to the mammary epithelium. *Msx-2* RNA was found in stroma closely associated with epithelial elements, not in epithelial cells and not in stroma distant from epithelial elements. In pregnancy, *Msx-1* expression was detected in epithelium of the ducts as well as the developing alveoli. *Msx-2* expression was associated mainly with stromal cells surrounding ducts and not with cells in the less abundant stroma associated with lobule-alveolar structures. The close physical association of cells displaying *Msx-1* and *Msx-2* transcripts with mammary epithelium strongly suggests that these homeogenes play a role in the inductive interactions occurring between mammary epithelium and stromal cells of the mammary fat pad.

Msx-2 expression was not detected in epithelium-free fat pad. This indicates that mammary epithelium is required for the expression of *Msx-2* in contiguous periductal stroma. The absence of *Msx-2* expression in stromal cells associated with blood vessels indicates a degree of specificity in this tissue interaction. Interestingly, *Msx-1* expression and *Msx-2* expression, which are normally found in the mesoderm underlying the apical ectodermal ridge in developing chick limbs, are not maintained in a limbless mutant that is unable to form an apical ridge (Robert *et al.*, 1991). The mutant can be rescued by grafting normal ectoderm to the limb field, leading to expression of these homeobox genes. Our results indicate that mesodermal expression of *Msx-2* in the mammary gland also requires contiguous mammary epithelium. In the case of the breast, an additional regulatory element is indicated by the influence of endogenous estrogen on *Msx-2* expression.

ACKNOWLEDGMENTS

This research was supported by a grant from the U.S. Department of the Army DAMD 17-94-J-4230. We thank Dr. R. Hill for provid-

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ing us with *Msx-1* and *Msx-2* clones. We also thank Phyllis Strickland, Dr. Gary Silberstein, and Kathy Van Horn for their assistance.

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Received for publication December 28, 1995
Accepted March 19, 1996

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